IRRADIANCE AND ABSCISIC ACID EFFECTS ON ACCLIMATIZATION OF MICROPROPAGATED *ARONIA ARBUTIFOLIA* (L.) ELL

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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This work is dadicated to my daughter Veronica and to my mother Josefina.

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Abscisic acid (ABA) was applied in vitro to ascertain its possible role on developmental and physiological changes of Aronia arbutifolia microcuttings grown under in vitro conditions and transplanted to contrasting irradiance levels.

Exogenous ABA treatments caused growth inhibition of microcuttings under in vitro conditions. Both shoot and root growth were reduced by the addition of ABA to the medium. Examination showed alterations in the leaf surface morphology of ABA treated plants. Abscisic acid at 4 mg/L or more induced epicuticular wax depositions on the abaxial surface of the leaves. These leaves then morphologically resemble those of greenhouse grown plants. Following transplant out of culture (ex vitro), growth of ABA treated microcuttings was reduced compared to the control. Net carbon assimilation (A)

of in vitro grown microcuttings was negative. Ex vitro A was lower for ABA treated microcuttings than for control plants. The effects of exogenous ABA treatments persisted up to 10 days post transplant ex vitro. At 20 days post transfer, growth and A were similar in all treatments and controls.

Nonstructural carbohydrate content in leaf and stem tissue of microcuttings was altered with ABA treatments. Under in vitro conditions and after 5 days of ex vitro growth, leaf soluble sugar content was higher in ABA treated plants than controls. Leaf chlorophyll, carotenoid, and leaf water loss were unaffected by the ABA treatments applied in vitro.

Under ex vitro conditions, rooted microcuttings were placed under two irradiance treatments of 450 and 650 μmol m^{-2} s^{-1}. The higher irradiance levels caused a reduction in overall growth, while nonstructural carbohydrate content was greater than at 450 μmol m^{-2} s^{-1}. Light compensation points for rooted microcuttings grown at 450 were lower than at 650 μmol m^{-2} s^{-1}.

In summary, in A. arbutifolia microcuttings, endogenous ABA played an important role in leaf wax development and increased total non-structural carbohydrates of the leaves compared to control plants. When applied in vitro, this growth regulator has the potential of pre-hardening the leaf prior to the onset of water stress encountered at transplanting into an ex vitro environment.

CHAPTER 1
GENERAL INTRODUCTION

The term acclimatization is used to define the process by which organisms adapt to man-made environments (Grainerd and Fuchigami, 1981). Irradiance, temperature, relative humidity, and growth regulators have a direct effect on acclimatization These factors are modified in artificial growing environments in order to achieve maximum plant growth and development. Yet, under most situations, economical considerations hinder the implementation of optimum levels of these factors The grower must then decide which factors should be altered in order to obtain optimum plant growth and development in an economically feasible manner.

Micropropagation is a tool which is employed in plant breeding (Kane et al., 1987), commercial plant production (Jones, 1987), genetic conservation (Jarrett and Gawel, 1991), and basic plant science research (Brooms and Zimmerman, 1978, Hasegawa, 1979). One of the main limitations with micropropagation has been related to the problems encountered with transplanting microcuttings from the laboratory to the greenhouse environment (Sutter, 1988; Pools and Conover, 1983;

Pierik, 1988) Microcuttings undergo morphological and physiological changes which provide them with an adaptation to changes in irradiance, temperature, and humidity following transfer to the ex vitro environment.

The basic micropropagation protocol for woody plants consist of four Stages (Murashige 1974) as summarized in Figure 1-1. Stage I is the establishment of the aseptic culture. The main objective of Stage I is to ensure that the plant material is completely free from microbial contaminants and that rapid growth can be obtained on the particular growing medium. Stage II is the multiplication step in which a higher amount of cytokinin to auxin is added to the growth medium, higher than in other Stages, in order to enhance axillary shoot initiation and subsequent rapid increase of organs. Stage III occurs when these microcuttings are prepared for reestablishment in the greenhouse. The medium of choice is supplemented with additional auxins for stimulation of root growth. Finally, during Stage IV, rooted microcuttings are transferred from the laboratory to the greenhouse. In this stage, rooted microcuttings transform from a heterotrophic to autotrophic state.

Environmental factors differ greatly between the in vitro and ex vitro growing conditions. Under in vitro conditions, microcuttings are growing under low irradiance (<75 μmol m^{-2} s^{-1}), moderate temperature (23-28 C), high

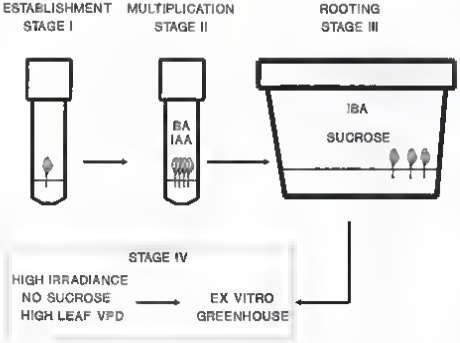


Figure 1-1 General schematic of the micropropagation protocol for a woody plant.

humidity (100%), and are dependent on sucrose in the media for their carbon source. Sucrose in the media and, to a lesser extent, low irradiance cause the plant to lack normal photosynthetic capacity and be totally heterotrophic (Grout and Millam, 1985; Langford and Weinwright, 1987, Capelladea et al., 1991).

Upon transplanting to an ex vitro environment, the microcuttings are induced to adapt to abrupt changes in environmental factors. These rooted microcuttings then have to adjust to a higher irradiance level, higher temperature, lower relative humidity, and changes in their method of carbon nutrition (Grout and Aston, 1978). A considerable amount of research has shown that microcuttings grown in vitro versus ex vitro, differ substantially in growth and development, leaf anatomy, morphology, and physiology as a direct result of environmental/cultural factors (Grainerd et al., 1981; Wetzstein and Sommer, 1983; Lee et al., 1985, 1988, Sutter, 1988). In micropropagation, problems associated with acclimatization occur when microcuttings are transferred from Stage III to Stage IV.

The contrasting in vitro and ex vitro growing environments is analogous to aquatic versus terrestrial growing conditions. Certain plant species have evolved to survive growing under both aquatic and terrestrial environments (Kane and Albert, 1989). Depending on the growing environment, these plants produce both submerged and

aerial leaves. Aleo, Salvucci and Bowes (1982), found aerial leaves of an amphibious plant to have much higher photosynthetic rates then submerged leaves. The question is then whether microcuttings can exhibit the same morphological and physiological plasticity as amphibious plants in response to modifications of environmental factors.

The following discussion will highlight previous research in which modification of environmental factors has been shown to affect plant growth and development.

Irradiance

Full sunlight can provide up to 2000 μmol m^{-2} s^{-1} of photosynthetic photon flux density, while in the greenhouse irradiance levels range from 360 to 1400 μmol m^{-2} s^{-1} (Conover and Poole, 1984). Photosynthetic photon flux density (PPFD), or irradiance as it will be termed in this study, is defined as the number of photons (400 to 700 nm) incident on a unit surface and per unit time (Shibles, 1975; Krizek and McFarlane, 1983). Irradiance is an integral component of the photosynthetic process. It provides the energy source for plants to convert radiant energy into chemical energy, eventually stored in organic molecules (Lawlor,1987).

Growth and Development

Growth reduction can occur as a result of high irradiance, caused by reducing enlargement of cells as a consequence of the perturbation of the balance of growth regulators (Levitt, 1980). High irradiance can also induce

water stress due to increased evaporation rates caused by the absorbed energy.

Low irradiance, on the other hand, may reduce plant growth due to low levels of non-structural carbohydrate accumulation. When plants are grown below their light compensation point, insufficient photosynthates are produced to maintain active growth. The light compensation point (LCP) is the irradiance level at which net carbon dioxide assimilation is equal to respiratory carbon dioxide evolution (Levitt, 1980).

Due to economical constraints, irradiance levels under in vitro conditions are kept low and fluctuate from 20 to 65 μmol m^2 s^1 resulting in slow growth (Smith et al , 1986; Kozai et al., 1988). Some studies have shown that manipulation of irradiance under in vitro conditions can lead to higher growth rates if sucrose levels in the media are reduced and gas exchange between the culture vessel and the outside atmosphere is maintained (Kozai et al., 1988a; Hayashi et al , 1988). Higher irradiance levels alone do not lead to increased growth rates, due to the lack of a normal photosynthetic apparatus (Grout and Aston, 1978) and to the low carbon dioxide levels prevalent in vitro (Desjardins et al., 1988).

Under ex vitro conditions, increasing irradiance levels have positive or no effects on growth and development. Desjardins et al. (1987) provided strawberry microcuttings with 150 μmol m^2 s^1 of supplemental lighting when natural

irradience levels were <250 μmol m^{-2} s^{-1}. Growth rates, dry matter accumulation, and total leaf area were substantially increased with supplemental lighting. Donnelly and Widaver (1984) also obtained higher leaf area of raspberry microcuttings with increasing irradiance levels. On the other hand, Lee et al., (1988) did not obtain higher leaf area or leaf thickness of sweetgum microcuttings grown at 315 versus 50 μmol m^{2} s^{1} irradiance

In general, during active plant growth periods, higher irradiance levels result in plants with shorter shoot length, decreased leaf area, and root growth, while, shoot and leaf dry weights are generally increased with increasing irradiance (Boardman, 1977; Longstreth et al , 1985).

Leaf Anatomy and Morphology

Leaf anatomical and morphological features are altered with increased irradiance Plants develop protective mechanisms for light avoidance, such as a thick cuticle, deposition of wax, and chemical protectors (Levitt, 1980)

Economou and Read (1986) increased number, length, and quality rating of new azalea (Rhododendron sp.) shoots produced at 30 to 70 μmol m^{-2} s^{1} irradiance than at 10 μmol m^{-2} s^{-1}. In another study, Economou and Read (1987) found the auxin content of 'White Lights' azaleas to increase progressively from 25 to 80 μmol m^{-2} s^{1} of irradiance. Other studies have dealt with the effects of irradiance in combination with carbon dioxide enrichment and have not shown

the isolated effects of irradiance on plant growth and development (Kozai et al., 1988; Figueira et al., 1991).

In leaves of micropropagated sweetgum, high irradiance (315 μmol m^{-2} s^{-1}) was associated with more compact mesophyll and smaller cells than low irradiance (50 μmol m^{-2} s^{-1}) (Lee et al., 1988). These same authors reported greater stomatal densities of microcuttings grown at higher irradiance.

Lee et al. (1985) compared chloroplast ultrastructure between micropropagated plants and seedlings of sweetgum. They found chloroplasts taken from leaves produced in vitro at 50, 155, and 315 μmol m^{-2} s^{-1} had disorganized grana at the lowest irradiance and lacked starch grains in all three irradiance treatments. Seedlings of the same species had abundant starch grains and disorganized grana at high irradiance; while at low irradiance they had fewer starch grains and well-formed grana. On the other hand, Capelladas et al. (1991) noted prominent starch grains in leaves of micropropagated rose at 50 μmol m^{-2} s^{-1}. In general, under high irradiance, leaves contain fewer chloroplasts with reduced pigmentation and less grana stacking (Boardman, 1977; Levitt, 1980; Anderson, 1986).

Leaf Physiology

Grout and Donkin (1987) noted lower photosynthetic rates of cauliflower microcuttings grown under in vitro environments than seedlings of the same species and found micropropagated leaves had low levels of chlorophyll and low ribulose

biephosphate carboxylase/oxygenase activity. This anomaly resulted in low A of micropropagated leaves when compared to leaves from seedlings. Higher irradiance treatment increased in vitro A if combined with carbon dioxide enrichment and reductions of the sucrose level in vitro (Langford and Wainwright, 1987; Kozai et al , 1988; Fujiwara et al., 1988, Capellades et al., 1991).

Plants acclimatized to low irradiance levels have lower light compensation points (LCP) than plants grown at higher irradiance Light acclimatization to low irradiance enables the plant to accumulate photosynthetic products at lower irradiance levels (Levitt, 1980). High irradiance acclimatization, on the other hand, increases the LCP and increases the light saturation level for net carbon assimilation (Bowes et al., 1972).

Leaf Biochemistry

No studies have dealt with correlating levels of nonstructural carbohydrates in microcuttings with irradiance levels under in vitro or ex vitro environments. Starch accumulates during illumination, while during darkness it is remobilized and consumed in respiration (Lawlor, 1987) High endogenous sucrose concentrations and plant growth are positively correlated with increased irradiance (Jenner, 1976, Gordon et al , 1980).

Temperature

Each plant species has an optimum growth temperature range for normal growth and development. The maintenance of optimum growth temperature in tissue-culture laboratories is the main consideration in choosing the irradiance source and level. Under artificial growing conditions a compromise is maintained by providing adequate irradiance levels without adversely increasing the heat load inside the culture vessels.

Evaporative Dehydration

Evaporative dehydration refers to both natural and artificial net water loss of water vapor due to exposure to a water (or drought) stress (Levitt, 1980). Any environmental factor that increases evaporative dehydration will result in increased water loss and possibly stomatal closure.

Growth and Development

Several studies have investigated the effects of low humidity in micropropagation acclimatization. Microcuttings of chrysanthemum cultured under low humidity had higher mortality rates and reduced number of roots (Wardle et al., 1983). Leaves from microcuttings have higher rates of water loss than greenhouse-acclimatized leaves (Brainerd and Fuchigami, 1981, Brainerd et al., 1981). These authors speculated that the higher rates of water loss of microcuttings resulted from higher cuticular conductance to water and to slow stomatal closing response.

Leaf Anatomy and Morphology

Water stress increases leaf wax development and deposition (Baker, 1974; Jordan et al., 1983). Fuchigami et al (1981) observed leaves from greenhouse-acclimatized (Prunus) to have more abaxial and adaxial epicuticular wax than microcuttings directly taken from in vitro. Wardle et al. (1983) reported reduced growth of micropropagated cauliflower, but they also noted large quantities of surface wax and low rates of water loss. Reduced relative humidity in the culture vessel caused more deposition of epicuticular wax, increased stomate density and reduced epidermal cell size of Rosa microcuttings (Capellades et al., 1990).

Leaf Physiology

No studies have investigated the effects of increased evaporative dehydration or water deficit on leaf physiology under in vitro conditions. However, in field grown plants, increases in water deficit causes either stomatal or non-stomatal inhibition of photosynthesis (Ward and Bunce, 1986).

Leaf Biochemistry

Soluble sugars were shown to accumulate in hypocotyls and roots of soybean seedlings exposed to water deficits (Creelman et al , 1990). These authors concluded that this increase contributed to osmotic adjustment.

Carbon Dioxide

Carbon dioxide enrichment can benefit plants by providing more substrate for assimilation and offsetting the effects of photorespiration (Lawlor, 1987). Several plant responses such as increased net carbon assimilation, increased growth rates, and increased water use efficiency have been caused by carbon dioxide enrichment (Allen, 1990).

Carbon dioxide may be limiting under in vitro environments and is implicated in reducing photosynthesis and inducing heterotrophic growing conditions (Desjardins et al., 1988).

The following discussion will highlight recent studies conducted on the effects of carbon dioxide enrichment on acclimatization of micropropegated plants.

Growth and Development

Carbon dioxide concentration has been recorded at 100 ppm at plant level in Stage III culture (Desjardins et al., 1988) Microcuttings grown using carbon dioxide enrichment under in vitro culture have high growth rates when compared to plants without (Kozai et al., 1988; Fujiwara et al., 1988; Desjardins et al., 1988, Figueire et al., 1991; Laforge et al., 1991) These same studies have shown that high irradience levels in combination with carbon dioxide enrichment have a synergistic effects on plant growth.

Under ex vitro conditions, carbon dioxide enrichment

enhanced growth of transplanted microcuttings of grapes (Lakso et al , 1986). Combining carbon dioxide enrichment and increased irradiance levels under ex vitro conditions also caused a synergistic effect on plant growth (Desjardins et al., 1987, 1990).

Leaf Anatomy and Morphology

In raspberry, carbon dioxide enrichment and high irradiance contributed to increasing stomatal density of in vitro microcuttings, while not affecting stomatal index (no. of stomata/(no. of stomata + no. of epidermal cells)) X 100 (Desjardins et al., 1988).

Leaf Physiology

Several studies have shown that in vitro plantlets are capable of maintaining normal A if the sugar content in the medium is reduced and carbon dioxide enrichment or low oxygen concentrations are provided in the vessel (Shimada et al., 1988, Kozai et al., 1988; Desjardins et al., 1988).

Abscisic Acid

Growth regulators are routinely used for regulation of morphogenesis of micropropagated plants. For example, auxin and cytokinin ratios determine the development of either shoots or roots (Tran Thanh Van, 1981). In few instances have growth regulators been used for the specific purpose of enhancing ex vitro acclimatization.

Growth and Development

In most cases the ratio of auxin to cytokinin is increased in Stage III to stimulate root growth which would then have an indirect effect on ex vitro establishment of microcuttings. Until recently, ABA had not been used as an in vitro growth regulator (Kane and Albert, 1989; Jarret and Gawel, 1991; Mohamed et al., 1991).

Endogenous ABA plays a role in controlling plant growth and development and alleviating water stress (McMichael and Hanny, 1977). For example, Creelman et al. (1990) applied ABA to soybean hypocotyls and recorded decreased plant growth rates. Jarret and Gawel (1991) established that exogenous ABA inhibits axillary bud and root development of in vitro cultured sweet potato. Root growth is also inhibited by exogenous ABA (Newton, 1977; Wetta et al., 1981). Other studies have shown that ABA can enhance growth. For example, Hall and McWha (1981) found that exogenously applied ABA initially inhibited growth, but that after a short lag, it increased the leaf and tiller numbers in wheat. Sen et al. (1989) also observed enhanced shoot morphogenesis in loblolly pine exposed to ABA.

Leaf Anatomy and Morphology

Abscisic acid treatments on wheat cause reduction in leaf size and stimulation of trichome formation on leaf surfaces (Quarrie and Jones, 1977; Hall and McWha, 1981). Abscisic acid has been noted to mediate water stress by inducing

changes in stomatal differentiation and leaf cuticle development in aquatic heterophyllic angiosperms (Kane and Albert, 1987a; 1989; Goliber, 1989). Further, Kane and Albert (1987b; 1989) found that ABA induced aquatic shoots of heterophyllous angiosperms to produce aerial leaves. The aerial leaves were also shown to differ significantly in anatomy and morphology from the submerged leaves.

Leaf Physiology

Exogenously applied ABA induces stomatal closure (Bouesiba and Richmond, 1976, Zeevart and Creelman, 1988) and possibly alters gene expression (Brey, 1985). Blake et al. (1990) noted that exogenous ABA provided short-lived antitranspirant action in black spruce seedlings. Generally, ABA does not limit the capacity for A, but causes a reduction in net carbon assimilation as a result of stomatal closure (Farquhar and Sharkey, 1982).

Leaf Biochemistry

Creelman et al. (1990) were unable to detect changes in sugar accumulation in soybean seedlings treated with exogenous ABA; while, Sharkey et al. (1985) have shown that ABA-treated leaves had lower starch and sucrose synthesis rates than non-treated leaves.

This review has shown that environmental factors are drastically different between Stage III and Stage IV growing conditions. With time, transplanted microcuttings adapt and survive in the new growing environment by altering leaf

developmental and physiological processes. The previous results presented, outlining the role ABA plays on plant growth and development, were the basis for the formulation of the hypothesis for this research. Abscisic acid may play a major role in developmental and physiological changes of microcuttings and these alterations can result in a pre-hardening effect. More importantly, these changes may provide the microcuttings with an added advantage when transferred from the in vitro to the ex vitro environment. In order to quantify any possible effects due to exogenous ABA on microcutting, measurements and observations were made on growth, development, leaf morphology, physiology, and biochemistry of the microcuttings under in vitro and ex vitro environments.

This study used the plant material, Aronia arbutifolia (L.) Ell. which is a native Florida woody plant with potential for use in revegetation efforts (Dehgan et al., 1989). This plant species is readily micropropagated and it provides an ideal model system to study environmental and growth regulator effects on acclimatization.

CHAPTER 2
RESPONSE OF MICROPROPAGATED *Aronia arbutifolia* TO EX VITRO IRRADIANCE LEVELS

Introduction

Irradiance is an integral component of the photosynthetic process and provides the energy source for plants to convert radiant energy into chemical energy (Lawlor, 1987). Under in vitro environments, microcuttings are exposed to low irradiance levels which vary from 20 to 65 μmol m^2 s^1 and result in slow growth (Smith et al., 1986; Kozai et al., 1988). Upon transplanting to the greenhouse at higher irradiance levels, growth rates increase significantly if microcuttings become acclimatized. Otherwise, high mortality levels occur due to the effects of decreased humidity (Brainerd et al., 1981).

Various researchers have shown that growth of tissue-cultured plantlets increases in vitro by increasing in vitro irradiance levels from 20 to 65 μmol m^2 s^1 to 120-150 μmol m^2 s^1 (Donnelly et al., 1985; Desjardins et al., 1987). Also, light saturation of an in vitro derived plant of sweetgum (*Liquidambar styraciflua*) ranged from 350 to 450 μmol m^2 s^1 (Lee et al., 1985) under ex vitro conditions.

Manipulation of greenhouse irradiance levels can enhance the acclimatization of transplanted microcuttings (Capelladas et al., 1990; Watzstein and Sommer, 1983). Desjardins et al. (1987) obtained higher leaf and root dry weights and leaf areas when strawberry microcuttings were placed in a greenhouse environment where the natural light level of <250 µmol m^2 s^1 was supplemented with 150 µmol m^2 s^{-1} from high intensity discharge lighting versus microcuttings without light supplementation. In a similar study, Desjardins et al (1990) showed that supplemental lighting of 80 µmol m^2 s^1 increased the dry weight of micropropagated asparagus after 22 days. Lee et al. (1988) grew sweetgum microcuttings under irradiance levels of 50, 155 and 315 µmol m^2 s^1. Plants grown under the highest irradiance had thicker leaves with greater mesophyll differentiation and larger cells than plants grown under low irradiance levels.

Microcuttings are produced under heterotrophic conditions, but once transplanted, they must shift to an autotrophic mode of nutrition (Grout and Aston, 1978) and hence, undergo changes in carbon nutrition. Smith et al. (1986) showed that plantlets achieved normal positive rates of photosynthesis within two weeks after transplanting.

Lee et al. (1985) observed tissue-cultured sweetgum plantlet chloroplasts had more grana development at 155 µmol m^2 s^1 and 315 µmol m^2 s^1 pretreatment irradiance levels compared to plants grown in vitro at 55 µmol m^2 s^1. At 55

μmol m^{-2} s^{-1} in vitro sweetgum plantlets contained chloroplasts with irregularly arranged internal thylakoid membranes.

This study was conducted to determine the developmental response of Aronia arbutifolia rooted microcuttings to ex vitro irradiance levels.

Materials and Methods

Stage I. Stems with lateral buds of A. arbutifolia were cut from actively growing and sexually mature plants, divided into 15-mm lengths with two to three lateral buds and rinsed in tap water for 1 hr. Lateral buds were surface sterilized by repeated immersion in 50% (v/v) ethanol for 1 min and then in 1.05% (v/v) sodium hypochlorite for 12 min, followed by three 5-min rinses in sterile deionized water (Kane et al., 1987). The sterilized nodal explants were transferred into 25 x 150 mm culture tubes containing 15 ml of medium consisting of Woody Plant Medium (WPM) salts and vitamins (Lloyd and McCown, 1980), 3% (w/v) sucrose, 1 mg/L N^6-benzylaminopurine (BA) and solidified with 1.0% (w/v) TC™ agar (JRH Biosciences, Lenexa, KS). The medium pH was adjusted to 5.5 with 0.1 N KOH before autoclaving at 1.2 kg cm^{-2} pressure for 20 min at 121 C. All cultures were placed under a 16-hr photoperiod provided by cool-white fluorescent lamps at 45 μmol m^{-2} s^{-1}. Air temperature was maintained at 25 $\pm$ 2 C.

Stage II. Stock cultures were maintained by subdividing shoots and transferring onto fresh medium every 5 to 7 weeks (Figure 2-1).

Figure 2-1. Stage II microcuttings of Aronia arbutifolia. Bar=10 mm

Stage III. Rooted microcuttings were prepared by cutting 5-week-old shoots into 10-mm stem segments consisting of two or three nodes. Fifteen microcuttings were transferred into 473-ml clear polypropylene culture vessels (Better Plastics, Kissimmee, Fla.) containing 100 ml of agar solidified WPM supplemented with 1 mg/L of indole-3-butyric acid (IBA).

Stage IV. After 30 days, Stage III rooted microcuttings (Figure 2-2) were transplanted into 15 trays of sterile Rootcubes (Smithers-Oasis, Kent, Ohio) containing 50 microcuttings/tray. Rooted microcuttings were fertilized weekly with a 20N-8.8P-16.6K soluble fertilizer (200 mg N/L). Trays were maintained in a walk-in growth room at 24 ± 2 C under Sylvania metal arc High Intensity Discharge (HID) lamps (GTE Products Corp., Manchester, N.H.) for a 16-hour photoperiod.

Rooted microcuttings were placed under three irradiance level-treatments (low: 350 μmol m^{-2} s^{-1}; medium: 550 μmol m^{-2} s^{-1}, high: 700 μmol m^{-2} s^{-1}). Irradiance attenuation was provided by polypropylene shade cloths. At transplanting, humidity domes were placed over the trays and progressively opened until fully removed at 10 days. Plants were watered as needed to maintain suitable substrate moisture.

At day 0 and after 30 days of ex vitro growth, fully expanded leaves from each treatment were collected for determination of net carbon assimilation (A) and chloroplast ultrastructure. Net carbon assimilation was determined with

Figure 2-2. Stage III rooted microcuttings of Aronia arbutifolia. Bar=10 mm.

the Hansatech D.W., Clark-type oxygen electrode (Hansatech Limited, Norfolk England). Immediately before measurement, leaves were cut and transferred to a leaf cuvette. Oxygen evolution was recorded using an oxygen electrode mounted in a cooling jacket (LD2, Hansatech) connected to a temperature controlled bath and light was supplied by a red diode light source (600 nm) Carbon dioxide was maintained at a stable level by adding a small amount 1 M Na_2HCO_3 to the mat under the leaf disc (Delieu and Walker, 1983). The leaf material was illuminated for 4 min at an irradiance of 450 $\mu mol\ m^{-2}\ s^{-1}$ and oxygen evolution was measured when steady state was reached.

Cytological examination of chloroplast development was made after 30 days post-transplant. Two leaf samples per replication per treatment were collected and fixed in 6% (v/v) glutaraldehyde, 6% (v/v) paraformaldehyde, 4% (v/v) osmium tetroxide and processed through an ethanol dehydration series (75-100%) prior to being embedded in Spurr's resin (Spurr, 1969). Resin blocks were heat polymerized and sectioned at 300 Angstrom with glass knives using a conventional ultramicrotome. Samples were viewed under a Jeol 100-CX transmission electron microscope at 65 kV.

Measurements on shoot length, total leaf area per plant, and total number of leaves were taken at 0, 10, 20, and 30 days after transplanting. Trays were arranged in a randomized complete block design with five replications. Blocking was implemented to counter the possible effects of an irradiance

gradient prevalent in the walk-in growth room. Five plants were chosen as subsamples per each replication. Data were analyzed by analysis of variance by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). First (linear) order polynomial was fitted to the data by regression analysis.

Results and Discussion

Post-transplant irradiance level of 700 μmol m^{-2} s^{-1} resulted in a 70% mortality rate, while plant survival was 100% at 350 and 550 μmol m^{-2} s^{-1}. At 10 days post transplant total leaf area decreased linearly with increased irradiance (Table 2-1). Shoot length, number of roots and number of leaves were not affected. After 20 days of ex vitro growth shoot length, total leaf area, number of roots, and number of leaves were not affected by irradiance treatments. At 30 days, shoot length, total leaf area, and number of leaves decreased linearly with increasing irradiance.

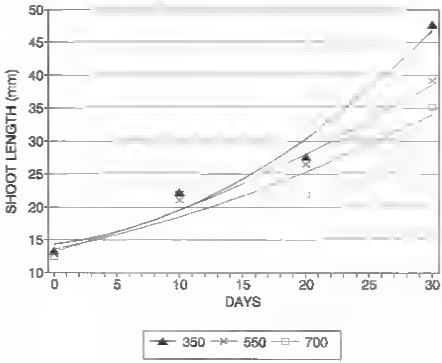
Compared to the 350 μmol m^{-2} s^{-1} treatment, irradiance treatments of 550 and 700 μmol m^{-2} s^{-1} caused shoot growth inhibition and slightly decreased the growth rate through time (Figure 2-3). At final harvest at 30 days, microcuttings maintained at 350 μmol m^{-2} s^{-1} had the longest shoot length.

Total leaf area per plant was also decreased by increasing irradiance levels. Rooted microcuttings have very small leaf area (approximately 0.76 cm^2) at time of transplant

Table 2-1. Growth and development of Aronia arbutifolia rooted microcuttings at three irradiance levels after 10, 20, and 30 days post-transplant.

	Shoot length			Leaf area			Roots			Leaves		
	Days post-transplant											
Irradiance	10	20	30	10	20	30	10	20	30	10	20	30
μmol m^{-2} s^{-1}		mm			cm^2			No.			No.	
350	22	28	42	1.0	1.3	7.5	4	6	6	5	5	7
550	21	27	36	0.7	1.8	6.4	4	6	7	5	5	6
700	22	25	33	0.6	1.3	5.4	3	5	6	5	4	5
Linear	NS	NS	**	**	NS	**	NS	NS	NS	NS	NS	**

NS,** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=5.



(350) $Y=14.3-0.242x+0.03x^2$ $r^2=0.97$

(550) $Y=13.2-0.531x+0.01x^2$ $r^2=0.98$

(700) $Y=13.5-0.405x+0.01x^2$ $r^2=0.90$

Figure 2-3. Shoot growth of transplanted rooted microcuttings of Aronia arbutifolia in response to three irradiance levels ($\mu mol\ m^{-2}\ s^{-1}$). Values are treatment means, n=5.

and maintain low values up to 10 days after transplanting, but leaf development rapidly increased at 20 and 30 days (Table 2-1). At 20 days post transplant the number of leaves produced per plant in the 700 μmol m^{-2} s^{-1} treatment decreased compared to the initial number of leaves per plant at day 0 (5.0 leaves per plant). This response was due possibly to photoinhibition caused by the increased irradiance level. At 30 days, the number of leaves produced was increased similarly in all treatments, indicating an acclimatization response to irradiance level had occurred (Table 2-1). The increase in shoot length and leaf area with decreased light intensity is in agreement with the behavior of sun and shade plants (Boardman, 1977). The growth reduction caused by the 550 and 700 μmol m^{-2} s^{-1} compared to 350 μmol m^{-2} s^{-1} was possibly due to disturbance of the balance of endogenous levels of growth regulators (Levitt, 1980; Economou and Read, 1987).

Total shoot dry weights and number of roots were similar at all irradiance levels after 10 and 20 days. Leaf area was reduced with increased irradiance while total shoot dry weights were not affected. These results were possibly due to the fact that sun leaves tend to have thicker leaves and higher fresh weight per leaf area than shade leaves (Longstreth et al., 1985; Boardman, 1977).

At day 0, net carbon assimilation averaged -7.76 μmol CO_2 m^{-2} s^{-1}. After 30 days post transplant, A was increased linearly from 12.3, 13.6, and 16.3 μmol CO_2 m^{-2} s^{-1} for the 450,

550, and 700 μmol m^2 s^1 irredience treatments, raapectively. Similar to rasulta obtained by Smith at al. (1986), the highest irradiance treatment had the highest A, probably due to modification of chloroplaat davalopmant (Andaraon, 1986)

Chloroplasts present in microcuttings cultured in vitro at 45 μmol m^2 s^1 prior to transplanting had disorganized thylakoid membranes with prominant starch granulaa (Figure. 2-4a). At 350 μmol m^2 s^1 and 30 daya poat transplant, thylakoid membranes were arranged in grana (Figure 2-4b). As the irradiance lavel increased from 350 μmol m^2 s^1 to 700 μmol m^2 s^1 grana atacking decreasad (Figure 2-4c,d). Chloroplaat daveloped undar higher light intensity of 700 μmol m^2 s^1 had laas grana atacking and may partition more stroma area for protein aynthaaia ao irradience energy may have been ueed more efficiently. Anderaon (1986) haa ahown that chloroplast devaloped under lower light intansity hava mora area partitioned for grana stacking, which increaaea the chlorophyll aontent for increasad capture of incoming irradiance but reduces the amount of carboxylation aubstratas needed for increased A.

Aa noted by other studies (Lee et al., 1985; Capellades et al., 1991), chloroplaata in tiaaua-culturad A. arbutifolia microcuttinga had diaorganized thylakoid davalopment undar an in vitro light intanaity of 45 μmol m^2 s^1 (Figure 2-4a). Thia anomaly could ba dua to tha limited gas exchanga and tha hatarotrophic mode of nutrition typical under in vitro

conditions (Grout and Aston, 1978). Starch granules were prevalent in chloroplasts from Stage III but no starch was observed in the ex vitro chloroplast. Two explanations are possible for the lack of starch in transplanted microcuttings: a) starch is more prevalent in high sucrose tissue culture medium and lower in ex vitro grown microcuttings or; b) under ex vitro conditions starch previously stored in vitro is converted to soluble sugars for use following transplant (Capelladas et al., 1991).

In this experiment, growth and development of transplanted rooted microcuttings were altered by light intensity. The best survival rate and growth response was obtained when transplanted rooted microcuttings were subjected to a post transplant irradiance level of 350 μmol m^2 s^1. Also, chloroplast development of in vitro cultured microcuttings were shown to be altered by the irradiance treatments.

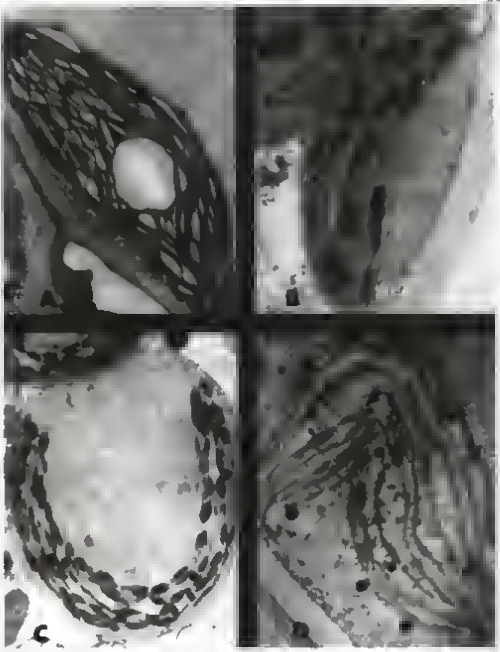


Figure 2-4. Chloroplast ultrastructure with grana of transplanted Aronia arbutifolia microcuttings (A. in vitro 45 μmol m^{-2} s^{-1}, 27,000X , B. ex vitro 350 μmol m^{-2} s^{-1}, 29,000X; C. ex vitro 550 μmol m^{-2} s^{-1}, 20,000X ; D. ex vitro 700 μmol m^{-2} s^{-1}, 29,000X). Electron micrographs are representative of two leaf samples per treatment.

CHAPTER 3
INFLUENCE OF ABSCISIC ACID AND IRRADIANCE ON GROWTH, DEVELOPMENT, NET CARBON ASSIMILATION AND NONSTRUCTURAL CARBOHYDRATE LEVELS OF *Aronia arbutifolia* MICROCUTTINGS

Introduction

Endogenous abscisic acid (ABA) plays a role in controlling plant growth and development and in alleviating water stress (McMichael and Hanny, 1977). Exogenously applied ABA inhibits plant growth (Creelman et al., 1990) by inducing stomatal closure (Boussiba and Richmond, 1976; Zeevart and Creelman, 1988) or possibly by altering gene expression (Gray, 1989). Under in vitro conditions, Jarret and Gawel (1991) established that exogenous ABA inhibits axillary bud and root development of in vitro cultured sweet potato. However, Sen et al., (1989) observed enhanced in vitro shoot morphogenesis in loblolly pine cotyledons exposed to ABA. Abscisic acid has also been noted to mediate water stress by inducing stomatal differentiation and leaf cuticle development in aquatic heterophyllic angiosperms (Kane and Albert, 1989; Goliber, 1989). Given that similar morphological changes occur when in vitro derived plants become acclimatized to ex vitro conditions, conceivably ABA could mediate the induction of these developmental changes.

Application of ABA ex vitro, increases the leaf and tiller numbers in wheat (Hall and McWha, 1991). These authors postulated that ABA caused a reduction in apical dominance. Exogenous ABA has also been implicated in causing senescence and loss of chlorophyll in leafy vegetables (Aharoni, 1979; Lipton, 1987) and reduction in leaf chlorophyll content when compared with control leaves (Popova et al., 1987).

During the acclimatization process, tissue cultured plantlets undergo changes in leaf morphology (Capellades et al., 1990) and physiology (Grout and Aston, 1978; Grout and Millem, 1995) which confer the plantlets with greater potential for survival ex vitro. For example, leaves of fully acclimatized plum microplants have greater cutinisation than leaves from microcuttings maintained in vitro (Brainerd et al., 1981). Sutter and Langhans (1982) showed that the lack of epicuticular wax on in vitro cultured plants contributed to water loss upon transfer to ex vitro conditions. However, Sutter (1985) cautioned that morphological differences in epicuticular wax between in vitro and ex vitro grown plants must be determined individually for each species under study.

Plant growth and development is affected by net carbon assimilation and transport of photoassimilates. Sucrose and starch are two major carbohydrates produced by the photosynthetic process. Under normal growing conditions, starch accumulates during illumination while it is remobilized and consumed in respiration during darkness (Lawlor, 1987).

Active plant growth creates a sink for sucrose and a subsequent decrease in photoassimilate partitioning into starch (Geiger, 1979). Abacisic acid can affect assimilation rates by decreasing net photosynthesis (Arteca et al., 1985) and diminishing the potential for fixation of atmospheric carbon dioxide into carbohydrates needed for growth and development (Sharkey et al., 1985)

A considerable amount of research has been conducted on the effects of irradiance levels on growth and development and whole plant physiology (Broadman, 1977). Highlights of these research findings have been determination of light compensation points (Fonteno and McWilliams, 1978; Faila et al., 1982) and chlorophyll content (Boardman, 1977; Kappel and Flore, 1983) as indicators of plant acclimatization to irradiance levels Irradiance has a direct effect on the photosynthetic supply of sucrose and may play a role in controlling translocation and partitioning at several steps (Gifford and Evans, 1981). Sucrose concentration and plant growth are both positively correlated with increased irradiance (Jsnner, 1978; Gordon et al., 1980). Nevertheless, while a substantial amount of research has concentrated on irradiance effects on leaf physiology, few studies have dealt with the effects of ABA on leaf physiology other then photosynthesis and transpiration.

Few studies have been undertaken to relate effects of

exogenous ABA on growth and development (Kane and Albert, 1989; Jarret and Gawel, 1991), and chlorophyll content of microcuttings at Stage III. No studies have investigated the effects of ABA on A and non-structural carbohydrate content of rooted microcuttings under in vitro and ex vitro conditions.

The objective of this study was to determine the role ABA may have in developmental and physiological changes of rooted microcuttings under in vitro and ex vitro conditions. This research is specifically geared, to charecterize changes in growth, development, net carbon assimilation (A), chlorophyll content, and non-structural carbohydrate content of Stage III rooted microcuttings of Aronia arbutifolia as influenced by exogenous ABA treatments.

Materials and Methods

Stage I. Stems with lateral buds of A. arbutifolia were cut from actively growing and sexually mature plants, divided into 15-mm lengths with two to three lateral buds and rinsed in tap water for 1 hr. Lateral buds were surface sterilized by repeated immersion in 50% (v/v) ethanol for 1 min and then in 1.05% (v/v) sodium hypochlorite for 12 min, followed by three 5-min rinses in sterile deionized water (Kane et al., 1987). The sterilized nodal explants were transferred into 25 x 150 mm culture tubes containing 15 ml of medium consisting of Woody Plant Medium (WPM), salts, and vitamins (Lloyd and McCown, 1980), 3% (w/v) sucrose, 1 mg/L N^6-benzylaminopurine

(BA) and solidified with 1.0% (w/v) TC™ agar (JRH Biosciences, Lenexa, KS). The medium pH was adjusted to 5.5 with 0.1 N KOH before autoclaving at 1.2 kg cm^2 pressure for 20 min at 121 C. All cultures were placed under a 16-hr photoperiod provided by cool-white fluorescent lamps at 45 $\mu mol\ m^2\ s^1$. Air temperature was maintained at 25 ± 2 C.

Stage II. Stock cultures were maintained by subdividing shoots and transferring onto fresh medium every 5 to 7 weeks.

Stage III. Rooted microcuttings were prepared by cutting five-week-old shoots into 10-mm stem segments consisting of two or three nodes. Fifteen microcuttings were transferred into 473-ml clear polypropylene culture vessels (Better Plastics, Kissimmee, Fla.) containing 100 ml of solidified WPM supplemented with 1 mg/L of indole-3-butyric acid (IBA). Synthetic ABA (90% mixed isomers, Sigma Chemical Co., St. Louis, Mo.) was prepared as a concentrated aqueous stock solution and sterilized by Millipore filtration (pore size: 0.22 μm) before being added to molten (40 C) sterile WPM medium at concentrations described in each of the following experiments. All cultures were placed under a 16-hr photoperiod provided by cool-white fluorescent lamps at 45 $\mu mol\ m^2\ s^1$. Air temperature was maintained at 25 ± 2 C.

Stage IV. After 30 days, Stage III rooted microcuttings were transplanted into trays of sterile Rootcubes (Smithers-Oasis, Kent, Ohio) containing 50 microcuttings/tray (Figure 3-1).

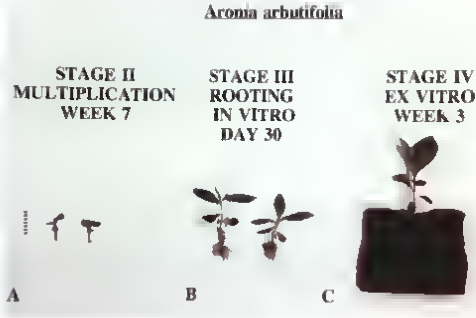


Figure 3-1: Aronia arbutifolia microcuttings at Stage II (A), III (B), and IV (C). Bar=10 mm.

Rooted microcuttings were fertilized weekly with a 20N-8.8P-16.6K soluble fertilizer (200 mg N/L). Trays were maintained in a walk-in growth room at 24 ± 2 C under Sylvania metal arc High Intensity Discharge (HID) lamps (GTE Products Corp., Manchester, N.H.) for a 16-hour photoperiod. Rooted microcuttings were placed under two irradiance treatment (low: 450 µmol m^{-2} s^{-1} and high: 650 µmol m^{-2} s^{-1}). Irradiance attenuation was provided by polypropylene shade cloths. At transplanting, humidity domes were placed over the trays and progressively opened until fully removed at 10 days. Plants were watered as needed to maintain suitable substrate moisture.

Experiment 1

A 5 X 2 factorial experiment was established with five levels of ABA (0, 0.1, 1, 10 and 100 mg/L) and two levels of IBA (0 and 1 mg/L) in Stage III culture. Culture vessels were arranged in a randomized complete design (RCD) with three vessels per replication. This first experiment was undertaken to define the effects of a wide range of ABA levels on the growth and development of A. arbutifolia microcuttings after 30 days of in vitro growth. Total shoot length, leaf area per plant, total number of roots per plant, total number of leaves per plant, and total dry weight were measured after 30 days of in vitro growth. Data were analyzed by analysis of variance by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). First (linear) or second (quadratic)

order polynomials were fitted to the data by regression analysis.

Experiment 2

Four levels of IBA (0, 1, 2 and 3 mg/L) were factorially arranged with four levels of ABA (0, 2, 4 and 6 mg/L) in Stage III culture in a randomized complete block design with five replications. Six plants were chosen as subsamples in each replication. The objective was to comprehend how IBA interacts with ABA in vitro and to determine if ABA alters leaf development. Total shoot length, leaf area per plant, total roots per plant and total number of leaves per plant were measured after 30 days of in vitro growth. In addition, first and second fully developed leaves closest to the apex, produced in vitro, were excised at the end of 30 days of in vitro growth and examined using scanning electron microscopy (SEM). Leaf samples were fixed in formalin-acetic-alcohol (FAA) for 24 hr then dehydrated through a graded ethanol series (25 to 100%), allowing 15 minutes in each solution. Dehydrated samples were dried in a critical point drier (Blazers CPD030), mounted on metal stubs and sputter coated (Sacsputter-2s, Tousimis Research Corporation, Rockville, Maryland) with gold. Samples were viewed with an Hitachi SEM-450 under 20 Kv.

Data were analyzed by analysis of variance by the General

Linear Model (GLM) procedure of the Statistical Analysis System (SAS). First (linear) or second (quadratic) order polynomials were fitted to the data by regression analysis.

Experiment 3

Abscisic acid levels of 0, 2, or 4 mg/L and 2 mg/L of IBA were applied in vitro in Stage III culture in a randomized complete block design with four replications under two irradiance treatments of 450 and 650 450 and 650 μmol m^2 s^1. Ten plants were chosen as subsamples for determination of shoot length, leaf area, number of leaves, and number of roots. Only two plants were chosen as subsamples for determination of carbon assimilation. The objective was to determine the short or long term effects of ABA on growth and A of rooted microcuttings. Shoot length, leaf area, number of leaves, number of roots, and A were determined at 30 days after in vitro growth. Abscisic acid treated (0, 2, and 4 mg/L) rooted microcuttings were also transplanted ex vitro at two irradiance levels of 450 and 650 μmol m^2 s^1. Measurements on shoot length, leaf area, number of roots and A were taken at 10, 20, and 27 days after transfer to the two irradiance treatments.

Net carbon assimilation was determined with the Hansatech D.W., Clark-type oxygen electrode (Hansatech Limited, Norfolk England). Immediately before measurement leaves were cut and transferred to a leaf cuvette. Oxygen evolution was recorded using an oxygen electrode mounted in a

cooling jacket (LD2, Hansatech) connected to a temperature controlling bath and light was supplied by a red diode light source (600 nm). Carbon dioxide was maintained at a stable level by adding a small amount of 1 M Na_2HCO_3 to the mat under the leaf disc (Delieu and Walker (1983). The leaf material was illuminated for 4 min at an irradiance of 450 µmol m^2 s^1 and oxygen evolution was measured when steady state was reached.

Data were analyzed by Analysis of variance by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). First (linear) order polynomial was fitted to the data by regression analysis.

Experiment 4

Abscisic acid was incorporated into the medium at 0, 0.5, 1, 1.5, 2, or 3 mg/L ABA and 2 mg/L IBA in Stage III culture in randomized complete block design with five replications. The objective was to determine if ABA and irradiance affect leaf development, biochemistry, and physiology. At the end of 30 days of in vitro growth and at 5, 10, 20, and 30 days post transplant, rooted microcuttings were assayed for non-structural carbohydrate content and chlorophyll content. Stomatal density, stomatal index, and water loss were only determined at the end of 30 days of in vitro growth.

Soluble sugars and starch content of leaf and stem tissue were extracted and analyzed with the phenol-sulfuric technique (Dubois et al., 1956). Whole plants were collected at 1500 hr and placed in a oven at 60 C. Dried leaf and stem material (0.01 g) was extracted with 8 ml of 80% (v/v) ethanol at 95 C for 20 minutes. After cooling, the samples were filtered through glass microfibre filters (Whatman International Ltd., Maidstone, England). The dried pellet recovered from the filtrate was hydrated with 2 ml of 0.1 M acetate buffer (pH 5.6) in a water bath at 85 C for approximately one hour. After cooling down to 37 C, 3 ml of enzyme solution (31 units alpha amylase ml^{-1} with 25.5 units of amyloglucosidase ml^{-1}, and 0.44 mg $CaCl_2$ ml^{-1}) was added and incubated in a shaking water bath for 24 hours at 37 C.

Samples were diluted to obtain a soluble sugar concentration between 15 and 25 μg ml^{-1}. An aliquot of 1 ml was taken and 1 ml of 5% phenol was added and agitated. Finally, 5 ml of concentrated sulfuric acid was added and the samples were placed for 20 minutes in a water bath at 25 C. Standard solutions of glucose ranging in concentrations from 10 to 70 μg ml^{-1} were prepared and treated in the same manner. The amount of soluble sugars present in the plant extracts were determined by optical density intensity assessed with a spectrophotometer (Perkin Elmer Lambda 3A, Perkin-Elmer Co., Norwalk, CT) at 490 nm.

Chlorophyll determinations were made following the procedure outlined by Bruinsma (1963). After 30 days of in vitro growth fully expanded leaves were excised, weighed, dried in a freeze drier, pulverized and added to 10 ml of 80% acetone. Solutions were placed in a refrigerator in the dark at 4 C for 24 hr, after which optical densities were measured at 663, 645, 652 nm for chlorophyll a, b, and total respectively. Carotenoid content was determined using a wavelength of 480 nm.

Leaf samples for stomatal density and stomatal index determination were fixed in formalin-acetic-alcohol (FAA) for 24 hr at room temperature (25 C) then rinsed in distilled water and submersed in lactic acid (85%) for one week at 70 C. Leaf samples were mounted on glass microscope slides and viewed with a light microscope at 40X. Abacisic acid levels of 0, 0.5, 1.5, and 2 mg/L were used for measurements for LCP taken at 5, 10, and 20 days after transfer to the two irradiance treatments.

Data were analyzed by analysis of variance by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). First (linear) or second (quadratic) order polynomials were fitted to the data by regression analysis.

Water loss was calculated by excising four fully expanded leaves per experimental unit, then proceeding to weigh them every 15 min for 2 hr. The slope of each regression line

obtained was then analyzed for significant differences (Sutter and Langhans, 1982).

The light compensation point (LCP) of fully expanded leaves of microcuttings were determined at 5, 10, and 20 days post-transplant, by measuring the net carbon assimilation with the Hansatech D.W., Clark-type O_2 electrode at 0, 100, and 200 µmol m^2 s^1 680 nm light. Light compensation points were determined using an asymptotic exponential equation (Boote and Jones, 1987)

Data were analyzed by analysis of variance by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). First (linear) or second (quadratic) order polynomials were fitted to the data by regression analysis.

Results and Discussion

Experiment 1

There was a significant effect on shoot length and total dry weight due to an ABA X IBA interaction (Table 3-1). Shoot length and dry weight reduction was more pronounced in the treatments without IBA than in the treatments with IBA. However, IBA did not have an effect on any of the growth variables. Root development differed between IBA treatments. Roots formed indirectly from callus in microcuttings treated with IBA versus direct adventitious formation in non-treated microcuttings. Increasing ABA levels reduced shoot length, leaf area per plant, number of roots per plant, number of leaves per plant, and total plant biomass (Table 3-1)

Table 3-1. Effects of ABA and IBA on growth and development of Aronia arbutifolia microcuttings after 30 days in vitro at 45 μmol m^{-2} s^{-1}. (Experiment 1).

ABA	Shoot length	Leaf area	Roots	Leaves	Dry wt.
mg/L	mm	cm^2	No.	No.	mg
NO IBA					
0	21.1	1.1	1.0	5	10
0.1	23.4	0.9	0.8	5	12
1.0	16.8	0.7	1.4	6	7
10	14.0	0.2	0	4	4
100	11.3	0.1	0	2	2
ABA-Linear	**	**	**	**	**
ABA-Quadratic	**	NS	NS	*	**
1 mg/l IBA					
0	16.5	0.5	1	5	8
0.1	20.8	0.8	2	6	9
1.0	15.3	0.3	1	3	6
10	12.2	0.2	0	3	5
100	10.8	0.1	0	2	2
ABA-Linear	**	**	**	**	**
ABA-Quadratic	*	NS	NS	NS	**
ABA X IBA	*	NS	NS	NS	*
IBA [t test]	NS	NS	NS	NS	NS

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=3.

Reduction in overall plant growth was evident in treatments with and without IBA (Table 3-1). Significant reductions in plant growth occurred at 10 mg/L or more ABA. Creelman et al. (1991) noted similar reductions in dry weight of soybean seedlings with increasing concentrations of ABA applied ex vitro. This study showed that exogenous ABA causes in vitro growth inhibition in a micropropagated woody plant, just as in micropropagated sweet potato (Jarret and Gawel, 1991).

Experiment 2

Interaction was noted between ABA X IBA for shoot length and number of leaves (Table 3-2). Increasing IBA had an antagonistic interaction with ABA resulting in greater shoot length than in the treatment without IBA. Growth of microcuttings with intermediate ranges of ABA (2, 4, and 6 mg/L) resulted in growth retardation of shoot, leaf area, number of roots, and number of leaves (Table 3-2). In the presence of IBA, percent rooting was not affected by the ABA treatments. The diminished production of roots observed (Table 3-2) without IBA may have contributed to a reduction in nutrient uptake from the medium and resulted in the lower growth obtained.

Results from research by Grout and Aston, (1978) and Capellades et al (1990) have shown that in vitro cultured microcuttings are not actively photosynthesizing. Therefore, ABA is probably not inhibiting growth as a result of reductions in A brought about by stomatal closure.

Table 3-2. Effects of ABA and IBA on growth and development of *Aronia arbutifolia* microcuttings after 30 days in vitro at 45 μmol m^{-2} s^{-1}. (Experiment 2).

Treatment		Shoot length	Leaf area	Roots[z]	Leaves
IBA	ABA				
mg/L		mm	cm^2	No.	No.
0	0	11	0.9	1(44)	5
0	2	10	0.4	0	4
0	4	10	0.4	0	4
0	6	10	0.5	0	4
1	0	13	1.1	5(82)	6
1	2	11	0.6	1(94)	4
1	4	10	0.5	1(79)	4
1	6	10	0.6	1(70)	4
2	0	14	1.2	7(82)	6
2	2	11	0.6	1(97)	5
2	4	11	0.4	1(79)	4
2	6	10	0.5	1(76)	4
3	0	13	1.0	5(88)	5
3	2	11	0.6	1(88)	4
3	4	11	0.4	1(94)	4
3	6	11	0.5	1(59)	4
Significant effects					
ABA-Linear		**	**	**	**
ABA-Quadratic		**	**	**	*
IBA-Linear		**	*	**	NS
IBA-Quadratic		*	*	*	NS
ABA X IBA		*	NS	NS	*

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=5. [z] Numbers in parenthesis refer to percent rooting

Under in vitro conditions, tissues readily absorb sugars from the media through the apoplast (Gifford and Evans, 1981) and assimilates are not limited (Capelladas et al., 1991). One possible explanation for ABA causing growth inhibition might be that it is altering cell wall extensibility through alterations in protein synthesis (Cleland, 1987; Bensen et al., 1988). However, controversy exists on the true extent of changes in protein synthesis brought about by applications of exogenous ABA (Bensen et al., 1988, Creelman et al., 1990).

Another explanation could be related to the interaction of plant growth regulators. Plant growth regulators, especially auxins, are known to induce rapid cell enlargement, elongation and, in cultured systems, may promote cell division (Cleland, 1987; Krikorian et al., 1987). A possible explanation for this growth reduction could be that ABA inhibits auxin promoted growth (Rehm and Cline, 1973) by interfering with the proton pump at the plasmamembrane (Reed and Bonner, 1974). Other researchers have found ABA to inhibit or to oppose the action of auxin-induced proton secretion and growth (Reed and Bonner, 1974; Raschke, 1975, Smith and Mansfield, 1982). The results obtained from this experiment indicate that ABA has a pronounced effect on growth and development of microcuttings under in vitro conditions.

The abaxial and adaxial leaf surface of microcuttings grown with 2 mg/L IBA and no ABA had smooth epicuticular wax deposition and spherical stomata (Figure 3-2a,b; Figure 3-3a,b) In contrast, plants grown with 2 mg/L IBA with 4 and 6 mg/L ABA showed heavy deposition of horizontal rodlets of epicuticular wax surface and elliptical stomata (Figure 3-2c,d,e,f). Media supplemented with ABA at 4 mg/L or greater resulted in production of leaves in vitro which morphologically resembled 30-day-old greenhouse-produced leaves (Figure 3-3c). However, the adaxial leaf surface of treated plants with 1 to 6 mg/L of ABA did not fully resemble the adaxial leaf surface of greenhouse grown plants which showed amorphous deposition of epicuticular wax (Figure 3-3d). Greenhouse grown leaves had trichomes, whereas trichomes were not visible in leaves produced in vitro (Figure 3-3c).

Kane and Albert (1985) reported increased cuticularization with exogenous ABA treatments in aquatic heterophyllous angiosperms. However, the present results are the first report of exogenous ABA inducing epicuticular wax deposition in a woody plant under in vitro conditions. These findings are significant since greater epicuticular wax deposition could result in lower cuticular water loss (Jordan et al. 1984). This developmental response could be very advantageous for microcutting establishment under ex vitro conditions. Jordan et al. (1983) suggested that an increase

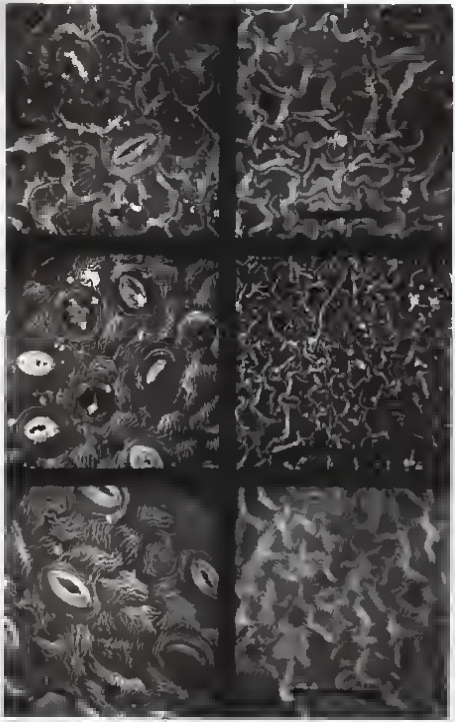


Figure 3-2. Leaf surface morphology Aronia arbutifolia microcuttings after cultured in vitro for 30 days (A. abaxial, B. adaxial, control: no ABA and 2 mg/L IBA, C. abaxial, D. adaxial, 4 mg/L ABA and 2 mg/L IBA; E. abaxial, F. adaxial, 6 mg/L ABA and 2 mg/L IBA), bar=5μ in A, C, and D and bar=50μ in B, D, and F. (Experiment 2). SEM representative of 3 leaves per replication.

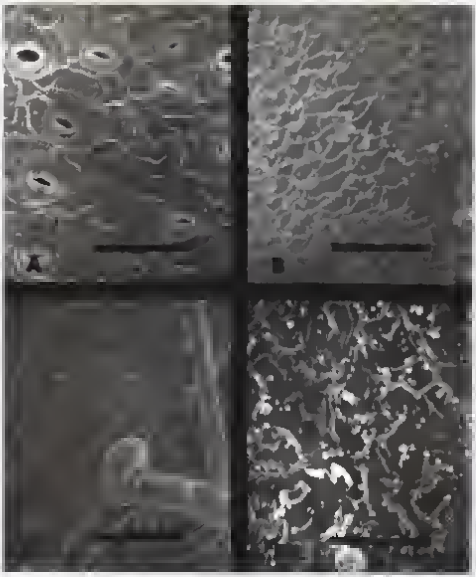


Figure 3-3. Leaf surface morphology of Aronia arbutifolia microcutting cultured in vitro (A, abaxial, B. adaxial) and grown in the greenhouse (C. abaxial, D adaxial), bar=50μ. (Controls).

in cuticle wax deposition occurs in response to water stress. In this experiment, no water stress was present among treatments. The results from this experiment clearly show, that in the absence of the environmental stimulus, ABA can enhanced leaf development similar to that observed during acclimatization.

Experiment 3

There was no interaction between ABA X irradience on growth, development, and A of microcuttings placed ex vitro. Microcuttings grown in vitro with media supplemented with ABA had reduced shoot length and leaf area compared to plants without ABA (Table 3-3). Number of leaves and roots, and A were reduced with exogenous ABA compared to the control. At this stage in vitro, A was low or negative ranging from 0.61 to -4.34 μmol CO_2 m^{-2} s^{-1}. Grout and Aston (1978) have shown microcuttings to have low or negative A under in vitro environments and attributed this response to low chlorophyll content and reduced ribulose biphosphate carboxylase activity. Capellades et al. (1991) also reported low A for micropropagated rose. These authors attributed this low A response to non-stomatal inhibition of photosynthesis caused by starch accumulation in the chloroplast of the rose leaves.

Growth reduction due to ABA treatments was evident at 10 days and up to 20 days after transplanting ex vitro (Tables 3-4 and 3-5). After 10 days, ex vitro plants previously grown with ABA supplemented in the in vitro medium had low or

Table 3-3. Effects of ABA on growth, development, and net carbon assimilation of Aronia arbutifolia microcuttings grown 30 days in vitro at 45 μmol m^{-2} s^{-1} (Experiment 3).

ABA Treatment	Shoot length	Leaf area	Leaves	Roots	Net carbon assimilation
mg/L	mm	cm^2	No.	No.	μmol CO_2 m^{-2} s^{-1}
0	16	1.1	3.1	11.7	0.61
2	14	0.8	2.6	11.5	-1.2
4	11	0.5	2.3	7.6	-4.34
ABA-Linear	**	**	**	**	**

NS, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=4.

Table 3-4. Effects of ABA on growth, development and net carbon assimilation of *Aronia arbutifolia* rooted microcuttings grown ex vitro under low and high irradiance for 10 days. (Experiment 3)

Irradiance	Treatment ABA	Shoot length	Leaf area	Roots	Net carbon assimilation
	mg/L	mm	cm^2	No	$\mu mol\ CO_2\ m^{-2}\ s^{-1}$
450 $\mu mol\ m^{-2}\ s^{-1}$					
	0	24	3.1	13	4.3
	2	20	2.2	11	3.3
	4	15	1.0	7	-0.3
ABA-Linear		**	**	**	**
650 $\mu mol\ m^{-2}\ s^{-1}$					
	0	21	2.7	13	2.7
	2	16	1.3	9	-3.7
	4	14	1.3	9	-3.6
ABA-Linear		**	**	*	**
ABA X Irradiance		NS	NS	NS	NS
Irradiance (t test)		NS	NS	NS	NS

NS,*,** Nonsignificant and significant at P = 0.05 or 0.01, respectivsly, n=4.

negative A levels ranging from 3 3 to -3 7 μmol CO_2 m^2 s^1, while microcuttings without ABA had regained positive A (Table 3-4). The ABA treated plants did not have positive A at 10 days of ex vitro growth (Table 3-4). At 20 days all ABA treated microcuttings had positive A rates (5.3 to 6.0 μmol CO_2 m^2 s^1) (Table 3-5). At 27 days (Table 3-6), A ranged from 4.7 to 5 6 μmol CO_2 m^2 s^1. These results indirectly confirm the occurrence of an after-effect of exogenous ABA on A. These results also suggest a resumption in normal leaf ABA balance probably occurred after 20 days. In the absence of ABA, A values were low or negative at day 0 and positive at 10 and 20 days in agreement with results of Grout and Millam (1985) showing that microcuttings depend on new leaves produced ex vitro to obtain higher A. In ABA treated explants at 10 days post transplant, an after-effect of exogenous ABA on A was probably due to decreased stomatal conductance (Dörffling et al., 1977; Arteca et al., 1985).

The results presented in this study differ to the results obtained by Branlard and Fuchigami (1982), in which they were unable to induce stomatal closure with exogenous ABA treatments. In their work, ABA was applied to excised leaves, while ABA was incorporated into the growing medium in this study, which may have resulted in increased ABA uptake. In this plant species and micropropagation method, A was lower in microcuttings treated with ABA and may have occurred as a result of decrease stomatal conductance.

Table 3-5. Effects of ABA on growth, development, and net carbon assimilation of Aronia arbutifolia rooted microcuttings grown ex vitro under low and high irradiance for 20 days. (Experiment 3).

Treatment		Shoot length	Leaf area	Roots	Net carbon assimilation
Irradiance	ABA				
	mg/L	mm	cm^2	No.	$\mu mol\ CO_2\ m^{-2}\ s^{-1}$
450 $\mu mol\ m^{-2}\ s^{-1}$					
	0	28	5.7	20	4.7
	2	21	3.5	13	5.5
	4	20	3.5	13	5.5
ABA-Linear		*	NS	NS	NS
650 $\mu mol\ m^{-2}\ s^{-1}$					
	0	27	5.8	17	5.1
	2	19	4.5	14	6.0
	4	18	2.3	13	5.3
ABA-Linear		**	**	NS	NS
ABA X Irradiance		NS	NS	NS	NS
Irradiance (t test)		NS	NS	NS	NS

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=4.

Table 3-6. Effects of ABA growth, development, and net carbon assimilation of Aronia arbutifolia rotted microcuttings grown ex vitro under low and high irradiance for 27 days (Experiment 1).

Irradiance / Treatment	ABA	Shoot length	Leaf area	Roots	Net carbon assimilation
	mg/L	mm	cm^2	No.	$\mu mol\ CO_2\ m^{-2}\ s^{-1}$
450 $\mu mol\ m^{-2}\ s^{-1}$					
	0	41	17.2	25	5.0
	2	32	11.0	21	5.6
	4	23	9.0	15	5.3
ABA-Linear		**	*	*	NS
650 $\mu mol\ m^{-2}\ s^{-1}$					
	0	33	12.0	21	4.8
	2	29	11.5	22	4.7
	4	27	11.0	22	5.0
ABA-Linear		NS	NS	NS	NS
ABA X Irradiance		NS	NS	NS	NS
Irradiance (t test)		NS	NS	NS	NS

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectivaly, n=4.

Sheckal et al. (1990) exposed micropropagated Malus (apple) shoots to 90% relative humidity and high boundary layer conductance and noted functional stomata using a modified steady state porometer and microscopic observations of stomatal aperture.

After 20 days under ex vitro conditions, plants grown with ABA had reduced shoot length and reduced leaf area (Table 3-5.). No differences were observed in A of ABA and non-ABA treated plants. After 27 days there were no residual effects of exogenous ABA on plant growth or net carbon assimilation. Irradiance did not have an effect on growth and development or net carbon assimilation at 10, 20, and 27 days after post-transplant (Tables 3-4, 3-5, and 3-6).

In a study with micropropagated strawberry, Mohamed et al. (1991) had obtained higher photosynthetic rates with exogenous ABA after 4 weeks of ex vitro growth. They stated that exogenous ABA had altered the juvenile expression of strawberry. Aronia arbutifolia did not undergo a visible juvenility phase, and A was not increased with ABA treatments.

In summary, the inhibitory effects of ABA were shown to occur independently of ex vitro irradiance levels and ABA was shown to reduce A and plant growth in the early stages of transplanting to ex vitro environments. The effects of this growth regulator were shown to be transient and diminish with time after ABA treatment was discontinued.

Experiment 4

After 30 days of in vitro growth, leaf soluble sugar levels were greater with ABA treatments than in the control, while leaf starch, and stem nonstructural carbohydrate content were not effected (Table 3-7). A likely explanation is that exogenous ABA mimics the effects of the onset of water stress in the leaves and results in an increase in leaf soluble sugars for osmotic adjustment (Henson, 1985). At this stage of leaf development the source of these solutes probably arises from the medium, since A is low or negative. Creelman et al. (1991) were unable to induce soluble sugar accumulation in the hypocotyl of ABA treated soybeans. They applied ABA as a spray treatment to the leaves, whereas in this study ABA uptake occurred through the stem. Henson (1985) noted greater uptake of ABA when applied to the shoot base of pearl millet than when applied directly to the leaf. The soybean seedling system described by Creelman et al. (1990) as well as the in vitro system used in this experiment cause minimal water stress on the test plants. Again exogenous ABA has been shown to mimic the effects of water stress and in this case alter carbon partitioning without the actual adversity of stress.

Furthermore, Kuang et al. (1990) reported osmotic adjustment to be linearly correlated with the reduction in leaf elongation rate in both wheat and lupin. Shoot length was shown to be reduced in ABA treated microcuttings as

Table 3-7. Effects of exogenous ABA level on soluble sugar and starch content of leaves and stems of Aronia arbutifolia microcuttings grown 30 days under in vitro conditions (Experiment 4).

ABA Treatment	Leaf		Stem	
	Soluble sugars	Starch	Soluble sugars	Starch
mg/L	mg/g DW			
0	7.4	17.9	9.8	22.1
1	9.4	15.6	10.6	21.9
2	10.6	14.7	13.1	20.9
3	11.8	17.9	11.9	19.3
ABA				
Linear	**	NS	NS	NS
Quadratic	NS	*	NS	NS

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=4.

compared to controls under both irradiance levels (Table 3-8).

There was interaction between ABA X irradiance for shoot length variable at 5 days post transplant. The 650 μmol m^{-2} s^{-1} irradiance level caused greater reduction in shoot length than 450 μmol m^{-2} s^{-1}. The onset of irradiance stress occurred quite rapidly once microcuttings were placed under the higher irradiance level (650 μmol m^{-2} s^{-1}).

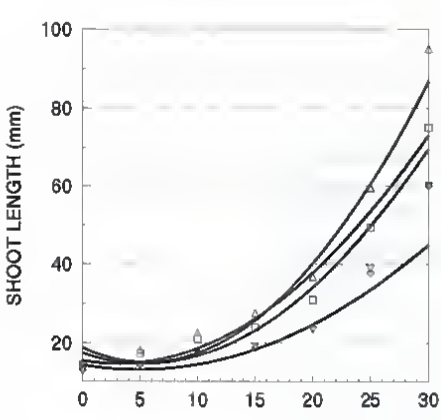
Shoot length of the control (no ABA) and the ABA treated microcuttings was inhibited by the increased irradiance treatment of 650 to a greater extent at 10 days and beyond compared to the lower irradiance of 450 μmol m^{-2} s^{-1} (Table 3-8). Retardation in shoot length due to exogenous ABA treatments was evident throughout the 30 day period of the experiment independently of irradiance treatment (Figures 3-4 and 3-5).

At 5 days after transfer ex vitro (Table 3-9), plants previously treated with ABA and grown at 450 μmol m^{-2} s^{-1} had higher levels of leaf soluble sugars than the control. On the other hand, leaf soluble sugars and stem soluble sugars were higher at 650 than at 450 μmol m^{-2} s^{-1} (Table 3-9). The higher irradiance level contributed to greater leaf and stem soluble sugar content than the lower level (Table 3-9). Prior ABA treatments did not have lingering effects on non-structural carbohydrate content after 10, 20, and 30 days post-transplant under both irradiance levels (Tables 3-10, 3-11, and 3-12).

Tabla 3-8. Effects of ABA on shoot growth of Aronia arbutifolia rooted microcuttings grown ex vitro under low and high irradiance for 5, 10, 15, 20, 25, and 30 days. (Experiment 4).

Treatment		Shoot length						
		Days post-tranaplant						
Irradiance	ABA	0	5	10	15	20	25	30
	mg/L	mm						
450 µmol m^{-2} s^{-1}								
	0	15	18	23	28	37	59	93
	1	14	17	21	24	30	49	75
	2	13	14	17	19	24	38	61
	3	13	14	17	19	23	39	61
ABA-Linear		*	**	**	**	**	**	**
ABA-Quadratic		NS	NS	NS	NS	NS	NS	NS
650 µmol m^{-2} s^{-1}								
	0	16	20	24	28	35	46	61
	1	12	13	16	17	20	30	44
	2	12	13	15	16	20	26	35
	3	11	12	15	16	20	29	41
ABA-Linear		**	**	**	**	**	**	**
ABA-Quadratic		*	**	**	**	**	**	*
ABA X Irradiance		NS	*	NS	NS	NS	NS	NS
Irradiance (t test)		NS	NS	*	*	*	*	*

NS,*,** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=5.



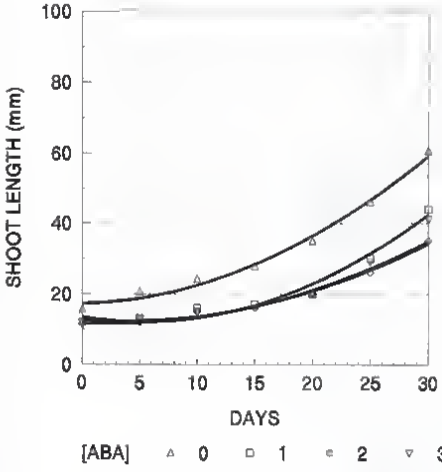
(0) Y=18.75-1.33X+0.12X^2 r^2=0.98

(1) Y=17.35-0.96X+0.09X^2 r^2=0.98

(2) Y=15.41-0.97X+0.08X^2 r^2=0.97

(3) Y=14.30-0.48X+0.05X^2 r^2=0.86

Figure 3-4 Shoot growth of transplanted rooted microcuttings of Aronia arbutifolia in response to four levels of ABA (mg/L) under an irradiance of 450 µmol m^{-2} s^{-1}. Values are treatment means, n=5 (Experiment 4).



(0) $Y=17.31+0.08X+0.04X^2$ $r^2=0.99$

(1) $Y=13.45-0.52X+0.05X^2$ $r^2=0.98$

(2) $Y=12.64-0.21X+0.03X^2$ $r^2=0.99$

(3) $Y=11.75-0.13X+0.03X^2$ $r^2=0.88$

Figure 3-5 Shoot growth of transplanted rooted microcuttings of Aronia arbutifolia in response to four levels of ABA (mg/L) under an irradiance of 650 µmol m^{-2} s^{-1}. Values are treatment means, n=5. (Experiment 4)

Table 3-9. Effects of prior ABA treatment and current irradiance level on leaf and stem soluble sugar and starch content of Aronia arbutifolia rooted microcuttings grown 5 days ex vitro. (Experiment 4)

Treatment		Leaf		Stem	
Irradiance	ABA	Soluble sugars	Starch	Soluble sugars	Starch
	mg/L	mg/ g DW			
450 μmol m^{-2} s^{-1}					
	0	9.0	17.2	6.0	38.1
	1	9.7	17.5	6.8	42.4
	2	13.4	21.3	9.5	47.2
	3	12.6	25.9	10.0	46.8
ABA-Linear		*	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
650 μmol m^{-2} s^{-1}					
	0	12.3	17.2	13.4	45.6
	1	12.8	22.6	12.8	32.7
	2	13.2	15.1	15.9	50.9
	3	15.1	20.7	15.6	42.3
ABA-Linear		NS	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
ABA X Irradiance		NS	NS	NS	NS
Irradiance (t test)		*	NS	*	NS

NS, * Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=3.

Table 3-10. Effects of prior ABA treatment and current irradiance level on leaf and stem soluble sugar and starch content of *Aronia arbutifolia* rooted microcuttings grown 10 days ex vitro (Experiment 4).

Treatment		Leaf		Stem	
Irradiance	ABA	Soluble sugars	Starch	Soluble sugars	Starch
	ng/L	mg/g DW			
450 μmol m^2 s^1					
	0	10.4	13.4	9.8	24.2
	1	10.7	15.2	9.4	24.4
	2	10.9	12.4	10.5	25.9
	3	13.6	20.9	11.4	27.9
ABA-Linear		NS	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
650 μmol m^2 s^1					
	0	11 7	12.6	14.5	42.1
	1	13.7	14.0	11.0	31.2
	2	12.9	12.4	16.2	40.6
	3	18 2	17.5	22.0	53.9
ABA-Linear		NS	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
ABA X Irradiance		NS	NS	NS	NS
Irradiance (t test)		NS	NS	*	*

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=3.

Table 3-11. Effects of prior ABA treatment and current irradiance level on leaf and stem soluble sugar and starch content of Aronia arbutifolia rooted microcuttings grown 20 days ex vitro. (Experiment 4)

Treatment		Leaf		Stem	
Irradiance	ABA	Soluble sugars	Starch	Soluble sugars	Starch
	mg/L	mg/g DW			
450 μmol m^{-2} s^{-1}					
	0	9.0	7.7	10.2	10.3
	1	9.7	9.0	10.1	13.2
	2	8.4	7.0	8.9	10.7
	3	8.0	8.1	10.1	16.4
ABA-Linear		NS	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
650 μmol m^{-2} s^{-1}					
	0	9.4	8.0	9.5	9.7
	1	9.5	7.9	11.1	16.3
	2	9.4	7.4	11.0	19.3
	3	9.6	8.3	10.3	15.8
ABA-Linear		NS	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
ABA X Irradiance		NS	NS	NS	NS
Irradiance (t test)		NS	NS	NS	NS

NS, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=3.

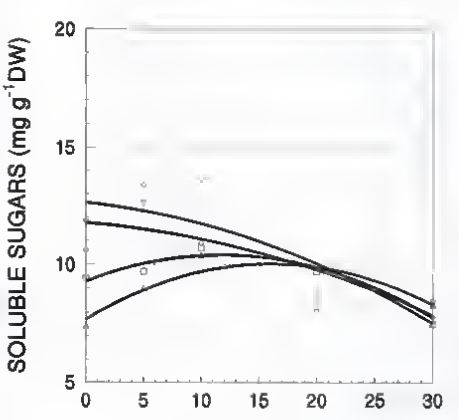
Table 3-12. Effects of prior ABA treatment and current irradience level on leaf and stem soluble sugar and starch content of *Aronia arbutifolia* rooted microcuttings grown 30 days ex vitro. (Experiment 4).

Treatment		Leaf		Stem	
Irradience	ABA	Soluble sugars	Starch	Soluble sugars	Starch
	mg/L	mg/g DW			
450 μmol m^{-2} s^{-1}					
	0	8.3	8.7	8.9	5.8
	1	7.5	8.3	9.5	5.9
	2	7.8	8.7	8.9	5.9
	3	8.4	7.2	8.9	8.2
ABA-Linear		NS	NS	NS	NS
Quadratic		NS	NS	NS	NS
850 μmol m^{-2} s^{-1}					
	0	9.2	6.3	10.1	8.8
	1	9.1	7.7	9.4	8.7
	2	8.4	8.9	10.2	7.7
	3	8.1	8.1	9.4	7.0
ABA-Linear		NS	NS	NS	NS
ABA-Quadratic		NS	NS	NS	NS
ABA X Irradience		NS	NS	NS	NS
Irradience (t test)		*	NS	*	*

NS, * Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=4.

Microcuttings grown at the higher irradiance level had greater stem soluble sugar and stem starch content at 10 and 30 days post-transplant than microcuttings at 450 μmol m^{-2} s^{-1} (Tables 3-10 and 3-12). At 30 days post-transplant, leaf soluble sugar content was greater under 650 than at 450 μmol m^{-2} s^{-1} (Table 3-12).

Immediately after transplanting microcuttings ex vitro, increased ABA levels had a greater effect on total non-structural carbohydrate (TNC) accumulation than did irradiance. After 10 days and beyond the higher irradiance level had a greater effect on TNC accumulation, while ABA did not. Shoot growth was negatively correlated with leaf TNC content, i.e., decreased shoot length with increased TNC content. Increase solute accumulation can result in increased osmotic adjustment (Tyree and Jarvis, 1982). As mentioned previously, Kuang et al. (1990) reported osmotic adjustment to be linearly correlated with the reduction in leaf elongation rate in both wheat and lupin. In this study, leaf soluble sugar content increased from day 0 (30 days in vitro) up to 10 days post-transplant. At 20 and 30 days, under both irradiance treatments, leaf soluble sugar content had decreased dramatically (Figures 3-6 and 3-7). These increases in leaf soluble sugar content at day 5 and 10, corresponded to limited shoot growth (Figures 3-4 and 3-5). However, decreased leaf soluble sugar content recorded at 30 days of ex



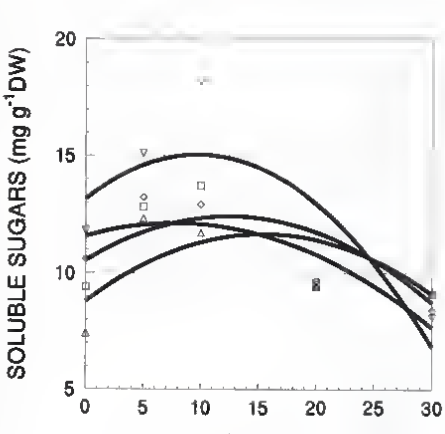
(0) $Y=7.70+0.29X-0.009X^2$ $r^2=0.73$

(1) $Y=9.28+0.19X-0.008X^2$ $r^2=0.96$

(2) $Y=11.77+0.04X-0.003X^2$ $r^2=0.67$

(3) $Y=12.63+0.05X-0.004X^2$ $r^2=0.64$

Figure 3-6. Influence of four levels of ABA (mg/L) on leaf soluble sugar content of *Aronia arbutifolia* rooted microcuttings during 30 days of ex vitro growth at 450 μmol m^{-2} s^{-1}, n=3.



(0) $Y=8.80+0.37X-0.012X^2$ $r^2=0.43$

(1) $Y=10.53+0.30X-0.012X^2$ $r^2=0.51$

(2) $Y=11.57+0.14X-0.009X^2$ $r^2=0.70$

(3) $Y=13.14+0.39X-0.020X^2$ $r^2=0.63$

Figure 3-7 Influence of four levels of ABA (mg/L) on leaf soluble sugar content of Aronia arbutifolia rooted microcuttings during 30 days of ex vitro growth at 650 µmol m^2 s^1, n=3.

vitro growth, was associated with increased shoot elongation under both irradiance levels.

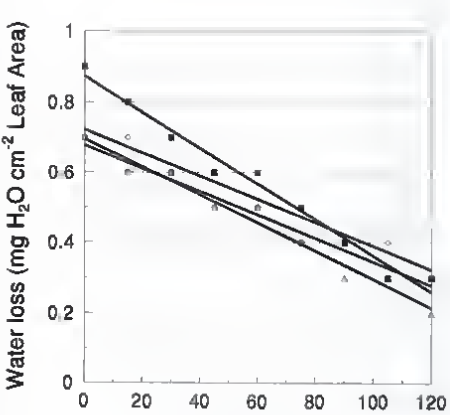
After 30 days of in vitro growth, no differences were found in stomatal index with increasing ABA levels compared to the control (Table 3-13). On the other hand, ABA treated plants exhibited a greater number of epidermal cells and increased stomatal density than the control. Abscisic acid treatments reduced overall cell size but did not increase formation of stomata. Newton (1977) calculated a 60% reduction in maximum cell elongation in Lemna roots with 2 mg/L of ABA. In terms of leaf area, this same author reported a 60% reduction between the treatment and the control.

After 30 days of in vitro growth, there were no differences between slopes for leaf water loss between the control and ABA treated microcuttings (Figure 3-8). Leaf water loss was not affected by increasing ABA levels, possibly due to using excised leaves in which stomata close rapidly and most of the water loss comes from cuticular conductance (Sutter and Langhans, 1982). Thus, water loss is a function of cuticular properties and may not be affected by ABA induced stomatal closure. A noticeable increase in epicuticular wax deposition was evident at 4 mg/L ABA or more. At 3 mg/L, leaf surface morphology was not altered. In order to diminish water loss, higher levels of ABA must be used. This creates a situation in which growth of microcutting would be compromised in gaining reduced cuticular conductance.

Table 3-13 Stomatal index of *Aronia arbutifolia* microcuttings grown in vitro for 30 days. (Experiment 4).

ABA Treatment	Stomatal density	No. epidermal cells	Stomatal index[z]
mg/L	mm^2	mm^2	mm^2
0	56	228	19.5
1	80	283	22.0
2	78	297	21.1
3	76	329	18.8
ABA-Linear	**	**	NS
ABA-Quadratic	**	NS	**

[NS, **] Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=10. [z] Stomatal index=No. of stomata/(No. of stomata + No. of epidermal cells)*100.



(0) Y=0.861-0.00486X r^2=0.95

(1) Y=0.691-0.00347X r^2=0.88

(2) Y=0.680-0.00369X r^2=0.86

(3) Y=0.723-0.00341X r^2=0.72

Figure 3-8. Effects of ABA on water loss (mg H_2O/cm^2 Leaf Area) of Aronia arbutifolia leaves excised from microcuttings grown in vitro for 30 days at 45 μmol m^2 s^{-1}. $t_{b\ calculated}$=0.2857 $t_{b\ 4df,.05}$=2.306. (Experiment 4)

Microcuttings had a higher light compensation point under the higher irradience level (Table 3-14). These results are in agreement with normal acclimatization of plants to irradiance (Fonteno and McWilliams, 1978; Falls et al., 1982). However, at 20 days post transplant, LCP was no longer affected by irradiance treatment. Obviously, all microcuttings had become equally light acclimatized independently of ABA treatment. It can be concluded that ABA did not have any effect on LCP. After prolonged exposure to the ex vitro irradiance levels, it seems the range between irradiance treatment was not enough to cause differences in LCP.

In summary, overall growth was diminished through the whole range of ABA levels tested. Exogenous ABA did not alter leaf physiology of A. arbutifolia rooted microcutting. Abscisic acid was shown to significantly alter leaf development, A, and non-structural carbohydrate partitioning. In terms of light acclimatization ABA did not affect chlorophyll content, and carotenoid content (Appendix) of rooted microcuttings under in vitro environments.

Compared to 450, the 650 μmol m^{-2} s^{-1} irradiance treatment affected LCP in the early stages of transplant and affected growth in the latter stages, but microcuttings were able to become physiologically acclimatized to the high irradiance treatment after prolonged exposure.

Table 3-14. Light compensation points of *Aronia arbutifolia* rooted microcuttings grown *in vitro* for 5, 10, and 20 days under 450 and 650 μmol m^{-2} s^{-1}. (Experiment 4).

Treatment		Light Compensation Point		
Irradiance	ABA	Day 5	Day 10	Day 20
	mg/L		μmol m^{-2} s^{-1}	
450 μmol m^{-2} s^{-1}				
	0	41	56	71
	0.5	58	32	50
	1.0	20	37	53
	1.5	61	60	53
	2.0	41	32	49
ABA-Linear		NS	NS	NS
ABA-Quadratic		NS	NS	NS
650 μmol m^{-2} s^{-1}				
	0	124	51	75
	0.5	120	60	67
	1.0	51	60	62
	1.5	64	63	62
	2.0	81	58	47
ABA-Linear		NS	NS	NS
ABA-Quadratic		NS	NS	NS
ABA X Irradiance		NS	NS	NS
Irradiance (t test)		*	*	NS

NS, *, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=5.

CHAPTER 4
CONCLUSIONS

Rooted microcuttings of Aronia arbutifolia were shown to respond to ex vitro irradiance levels. Overall, increasing ex vitro irradiance levels caused a marked reduction in growth and development. Grana stacking increased with decreased irradiance levels. This response in chloroplast development was correlated with decreased net carbon assimilation (A). Microcuttings were able to survive being transferred from a 45 μmol m^{-2} s^{-1} in vitro irradiance level to 350, 450, 550, and 650 μmol m^{-2} s^{-1} ex vitro irradiance, but did not adequately survive 700 μmol m^{-2} s^{-1}. Aronia arbutifolia was shown to adapt to increasing irradiance levels by increasing TNC content of leaves and stems. This biochemical alteration probably contributed to survival of microcuttings when transferred from a low in vitro irradiance level (45 μmol m^{-2} s^{-1}) to relatively high ex vitro irradiance levels of 350, 450, 550, and 650 μmol m^{-2} s^{-1}.

Endogenous ABA may have an important role in mediating acclimatization of Aronia arbutifolia microcuttings. Microcuttings previously treated with ABA under in vitro

conditions had greater leaf wax deposition, a response resembling greenhouse grown leaves. Abscisic acid mediates this leaf developmental response without the occurrance of an environmental stress. This procedure may prove beneficial for the establishment of sensitive microcuttings in ex vitro environments. An added advantage of ABA deals with possibly maintaining decreased stomatal aperture of microcuttings throughout the transition period (up to 10 days) from in vitro transfer to ex vitro establishment. At this stage, it is paramount that leaves produced in vitro can survive long enough for new leaf development to take place.

In Aronia arbutifolia the advantages of utilizing ABA for increased light acclimatization, and increased survival did not materialize. This lack of response was probably due to the natural plasticity of this plant species. However, ABA did induce biochemical changes which could potentially contribute to the survival of microcuttings when transplanted from one environment (in vitro) to another more extreme environment (ex vitro).

Based on this research, exogenous ABA treatments applied in vitro could be used as a means of pre-hardening of problematic species which encounter difficulties in ex vitro establishment.

APPENDIX

Table 1. Effects of ABA on chlorophyll a, b, total, and carotenoid content of *Aronia arbutifolia* rooted microcuttings after 30 days in vitro at 45 μmol m^{-2} s^{-1} (Experiment 4)

ABA Treatment	Chl. a	Chl. b	Total chl.	Carotenoids
mg/L	mg/g FW	mg/g FW	mg/g FW	nmol/g FW
0	0.65	0.44	1.35	38
1	0.59	0.46	1.35	36
2	0.69	0.46	1.44	40
3	0.56	0.34	1.08	27
Significant effects				
ABA-Linear	NS	NS	NS	NS
ABA-Quadratic	NS	NS	NS	NS

NS, ** Nonsignificant and significant at P = 0.05 or 0.01, respectively, n=3.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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